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The demonstration that plants can activate chemicals into mutagens raises the concern that plants might activate environmental agents and introduce genotoxins into the human food chain. In this project period we concentrated on the follwing research objectives. 1) The comparison of the plant activation of specific mono- and polycyclic aromatic amines (2-aminofluorene and m-phenylenediamine) by cultured plant cells and fresh water algae. 2) The investigation of the biochemical mechanisms of plant activation by the use of specific enzyme inhibitors. 3) The determination if specific inhibitors that constrain the activation of the substrates function by competitive or noncompetitive inhibition. We observed that 2-aminofluorene is a more potent promutagen than m-phenylenediamine. As little as 25 nmol 2-aminofluorene/reaction tube caused a significant increase in mutant TA98 cells. *S. capricornutum* did not activate m-phenylenediamine at concentration ranges similar to those used for the TX1 studies, although 2-aminofluorene was weakly activated. Both agents appeared to be non-toxic at the highest concentrations, based on microscopic observation of the algal cells after exposure to the chemicals. Under the conditions tested, it was

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concluded that the enzyme system(s) responsible for the activation of these chemicals in tobacco cells is either at low concentrations or inactive in the algal cells. The activation of 2-aminofluorene by TX1 cells was governed by an enzyme system(s) that was inhibited by diethyldithiocarbamate or 7,8-benzoflavone. (+)-Catechin (at low concentrations) or methimazole enhanced the activation of 2-aminofluorene while higher concentrations of (+)-catechin were inhibitory. A pathway of the plant activation of 2-aminofluorene is via a cytochrome P-448-dependent N-hydroxylase. Peroxidase may be a minor pathway. The activation of m-phenylenediamine was inhibited by diethyldithiocarbamate, (+)-catechin, methimazole, potassium cyanide, and acetaminophen. Inhibition by methimazole indicates the presence of a FAD-dependent monooxygenase in the activation of m-phenylenediamine. The inhibition of the plant activation of m-phenylenediamine by potassium cyanide and acetaminophen indicates that peroxidases may function as a major pathway. Diethyldithiocarbamate was a potent inhibitor of tobacco cell peroxidase activity under *in vivo* and *in vitro* conditions. Kinetic experiments demonstrated that diethyldithiocarbamate was a non-competitive inhibitor of TX1 cell peroxidase. One mechanism for the antimutagenic effect of diethyldithiocarbamate was through its inhibition of cellular peroxidases.

Report AFOSR-88-0336

THE MECHANISMS AND EFFECTS OF THE PLANT ACTIVATION OF CHEMICALS IN THE ENVIRONMENT

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Report for Period 20 September 1989 to 20 March 1990

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ANNUAL TECHNICAL REPORT

1   INTRODUCTION
1.1   The Risk of Environmental Mutagens

Environmental mutagens are recognized as serious threats to the public health. An environmental mutagen or genotoxin is an agent that is released into the environment that can alter the genetic material or alter the proper functioning of the genetic material. Depending upon the developmental stage of an individual, a genotoxin can exert teratogenic effects, precipitate coronary disease, produce mutations involving germinal cells, or cause mutations in somatic cells that may become neoplastic. It is widely believed that a majority of human cancers is due to the presence of carcinogens in the environment. The somatic cell mutation theory of cancer revolves around the simple premise that cancer arises in humans and other organisms through damage to DNA, chromosomes or to mitotic recombination. During the last decade, evidence has been reported which demonstrates that most carcinogens are mutagens (Crow and Abrahamson, 1982). Research conducted in the United States, Japan and Great Britain indicate a high correlation between carcinogenicity and mutagenicity ($r = 0.85$-0.93) indicating that environmental mutagens play a significant role in the induction of human cancer (de Serres, 1979).

1.2   Involvement of Plant Activation in Genetic Toxicology

The U.S. Environmental Protection Agency funded the first research on the plant activation of pesticides into mutagens (Plewa and Gentile, 1982; 1984; Gentile and Plewa, 1982). We reported the first direct evidence of the plant activation of an environmental agent (the s-triazine herbicide, atrazine) (Plewa and Gentile, 1976; Means ct al., 1988). Plant activation is the process by which a promutagen is activated into a mutagen by a plant system (Plewa, 1978; Plewa and Gentile, 1982). A promutagen is a chemical that is not mutagenic in itself but can be biologically transformed into a mutagen. The demonstration that plants can activate promutagens raises the concern that plants might activate environmental agents and introduce mutagens into the human food chain. When one considers the broad spectrum of chemical agents and the magnitude of their use in modern agriculture, a basis for concern is warranted. We know that plants can metabolize xenobiotics into agents that may be toxic to components of the ecosystem. These plant-mediated metabolites may pose a hazard to human health by their passage via ground water or the food chain.

2   LIST OF OBJECTIVES

The original objectives of this project were:

- To compare the plant activation of specific mono- and polycyclic aromatic amines (2-aminofluorene and m-phenylenediamine) by cultured plant cells and fresh water algae.

- To investigate the biochemical mechanisms of plant activation by the use of specific enzyme inhibitors.

- To analyze the rates of mutagenic product formation (by gas-liquid chromatography and/or high performance liquid chromatography) as a function of substrate

concentration, plant cell density, whole cell protein, and specific growth stage of the cell suspension culture.

- To determine if specific inhibitors that constrain the activation of the substrates function by competitive or noncompetitive inhibition.

- To analyze any synergistic effects on plant activation induced by increasing peroxidases by the widely-used herbicide, atrazine.

3   STATUS OF RESEARCH EFFORTS

The research generated for each objective is presented under the heading that identifies the specific objective.

3.1   Comparison of the Plant Activation of Monocyclyic and Polycyclic Aromatic Amines Using Tobacco and Algae Cell Cultures

3.1.1   Plant Cell/Microbe Coincubation Assay

The assay is based on employing living plant cells in suspension culture as the activating system and specific microbial strains as the genetic indicator organism (Plewa et al., 1983). The plant and microbial cells are coincubated together in a suitable medium with a promutagen (Figure 1). The activation of the promutagen is detected by plating the microbe on selective media; the viability of the plant and microbial cells may be monitored as well as other components of the assay (Plewa et al., 1988).

Figure 1 Composition of a plant cell microbe coincubation reaction tube.

Long-term plant cell suspension cultures of tobacco (*Nicotiana tabacum*), cell line TX1 were maintained in MX medium, a modified liquid culture medium of Murashige and Skoog (1962). *Salmonella typhimurium* strain TA98 was the genetic indicator organism used (Maron and Ames, 1983).

A TX1 cell culture (the activating system in the coincubation assay) was grown at 28°C to late-log phase, and the cells were washed and suspended in MX⁻ medium. MX⁻ medium is a modified Murashige and Skoog (1962) liquid culture medium that lacks plant growth hormone. The fresh weight of the plant cells was adjusted to 100 mg/ml and stored on ice (≤ 30 min) until used.

An overnight culture of *S. typhimurium* was grown from a single colony isolate in 100 ml of Luria broth (LB) at 37°C with shaking. The bacterial suspension was centrifuged and washed in 100

Mm potassium phosphate buffer, Ph 7.4. The titer of the suspension was determined spectro-photometrically at 660 nm, was adjusted to 1×10^{10} cells/ml and the culture was placed on ice.

In the coincubation assay, each reaction mixture consisted of 4.5 ml of the plant cell suspension in MX^- medium, 0.5 ml of the bacterial suspension (5×10^9 cells), a known amount of the promutagen in $\leq 25 \mu l$ dimethylsulfoxide (Figure 1). Concurrent negative controls consisted of plant and bacterial cells alone, heat-killed plant cells plus bacteria and the promutagen, and both buffer and solvent controls.

These components were incubated at 28°C for 1 h with shaking at 150 rpm. After the treatment time, the reaction tubes were placed on ice. Triplicate 0.5 ml aliquots (approximately 5×10^8 bacteria) were removed and added to molten top agar supplemented with 550 μM histidine and biotin. The top agar was poured onto Vogel Bonner minimal medium plates, incubated for 48 h at 37°C, and revertant his^+ colonies were scored (Fi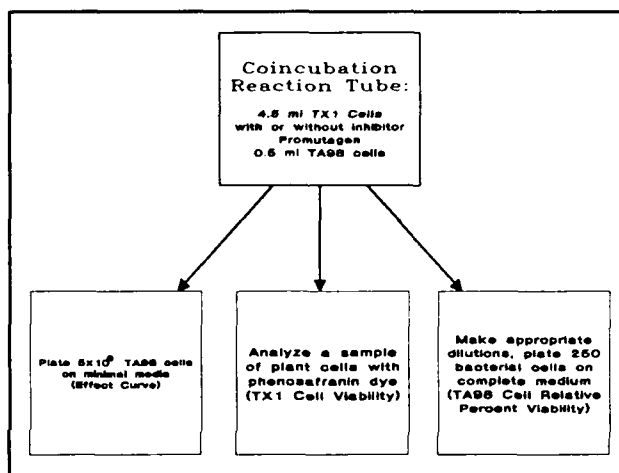gure 2). The remainder of the reaction mixture was used to determine the viability of the plant and bacterial cells. One volume of cold 250 mM sodium citrate buffer, pH 7, was added to each reaction tube which was then placed on ice. 0.5 ml of this suspension was removed and mixed with 2 ml of MX^- medium. The viability of the TX1 cells was immediately determined using the phenosaf-ranine dye exclusion method. The viability of the bacterial cells was determined by adding 1

Figure 2 Plating schedule and the analysis of the outputs from a reaction tube.

ml of the cold reaction mixture to 1 ml of cold 100 mM phosphate buffer, pH 7.4. A dilution series using phosphate buffer was conducted so that approximately 300 to 500 cells were added to each of three molten LB top agar tubes and poured upon LB plates (Figure 2). After incubation at 37°C for 24 to 36 h, the bacterial colonies were counted.

3.1.1.1 Activation of Aromatic Amine Promutagens by *N. tabacum* Cells

In our research we used the polycyclic and monocyclic aromatic amines, 2-aminofluorene (CAS # 153-78-6) and *m*-phenylenediamine (CAS # 108-45-2), respectively, as model promutagens. 2-Aminofluorene is a well characterized mammalian promutagen. The first stage in the mammalian activation of 2-aminofluorene is a *N*-hydroxylation reaction that is principally dependent upon the cytochrome P-450 enzyme system that functions as the terminal monooxygenase (Lotlikar and Zaleski, 1975; Nagata et al., 1985). 2-Aminofluorene is also activated by several plant species (Plewa et al., 1988). *m*-Phenylenediamine is an aniline derivative and is a promutagen in TA98 which is activated by rodent S9 (Ames et al., 1975; Shahin et al., 1983). *m*-Phenylenediamine is mutagenic in mouse L5178Y lymphoma cells (Palmer et al., 1977) and is a plant promutagen (Lhotka et al., 1987). Concentration-response curves for the TX1 cell activation of 2-aminofluorene (●) and *m*-phenylenediamine (■) are presented in Figure 3. The negative controls consisted of reaction tubes that contained the promutagen in MX^- medium plus TA98 without plant cells. As illustrated, 2-

aminofluorene is a more potent promutagen than *m*-phenylenediamine. As little as 25 nmol 2-aminofluorene/reaction tube caused a significant increase in mutant TA98 cells. Thus, the plant/cell microbe coincubation assay is a sensitive method to screen for plant-dependent promutagens.

3.1.1.2 Activation of Aromatic Amine Promutagens by *Selenastrum capricornutum* Cells.

Experiments were conducted to determine if *S. capricornutum* could activate 2-aminofluorene or *m*-phenylenediamine into a mutagen using *his$^+$* reversion in *S. typhimurium* strain TA98 as the genetic endpoint. A series of reaction tubes containing either 4.5 ml of MP medium or 4.5 ml of the algal cell suspension were set up. Concentrations ranging from 0-0.5 μmoles/reaction tube of 2-aminofluorene in no more than 50 μl dimethylsulfoxide and concentrations of *m*-phenylenediamine ranging from 0-10 μmol/reaction tube in no more than 100 μl dimethylsulfoxide were introduced. Concurrent controls containing either potassium phosphate buffer, MP medium, algal cells with no 2-aminofluorene or *m*-phenylenediamine, algal cells with the highest concentration

Figure 3 The activation of 2-aminofluorene and *m*-phenylenediamine by TX1 cells.

of DMSO with no promutagen, or *heat-killed* algal cells with 0.5 μmol 2-aminofluorene or 10 μmol *m*-phenylenediamine were assayed simultaneously. The reaction tubes were then incubated and treated as described in §3.1.1 except the algae and 2-aminofluorene or *m*-phenylenediamine were pre-incubated for 48 hr at 20°C with shaking in gold light prior to the plant cell/microbe coincubation assay.

The possibility that a primitive plant species, such as algae, could activate *m*-phenylenediamine and 2-aminofluorene was investigated using the plant cell/microbe coincubation assay with *S. capricornutum* as the activating

Figure 4 The activation of *m*-phenylenediamine and 2-aminofluorene by *Selenastrum capricornutum*

system. This species did not activate *m*-phenylenediamine (■) at concentration ranges similar to those used for the TX1 studies, although 2-aminofluorene (●) was weakly activated (Figure 4). Both agents appeared to be non-toxic at the highest concentrations, based on microscopic observation of the algal cells after exposure to the chemicals. Under the conditions tested, it was concluded that the enzyme system(s) responsible for the activation of these chemicals in tobacco cells is either at low concentrations or inactive in the algal cells.

3.2   The Investigation of the Biochemical Mechanisms of Plant Activation by the Use of Specific Enzyme Inhibitors
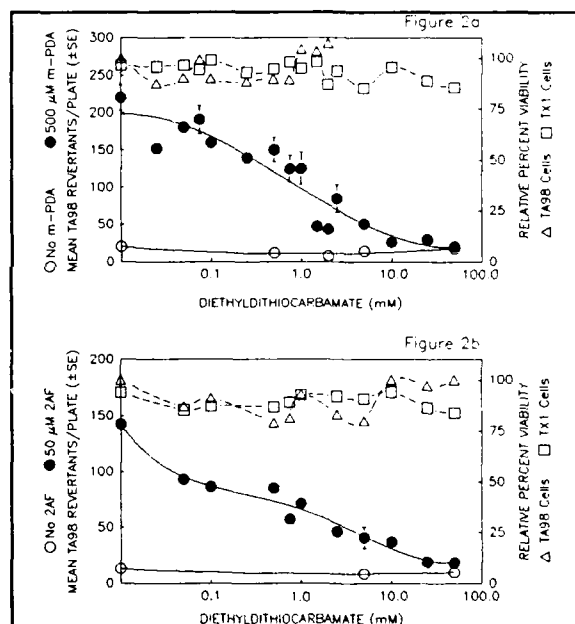
In this study seven inhibitors were analyzed for their ability to affect the plant activation of 2-aminofluorene or *m*-phenylenediamine. We also determined if each inhibitor was a direct-acting mutagen or a plant-activated promutagen. The endpoint of viability was included in the experimental design to investigate if the inhibitor, alone or in combination with plant cells (TX1 cells) and/or the promutagen was toxic to the activating system or to the genetic indicator organism (TA98 cells) (Figure 2). Viability is crucial for data interpretation; a typical inhibition curve with decreasing numbers of TA98 revertants with increasing "inhibitor" concentrations could be due to toxicity in the plant cells, toxicity in the bacterial cells, toxicity in both cell types or a true inhibition of plant cell activation. Thus with viability as an endpoint, significant alteration of the reversion frequency of TA98 could be interpreted as a true amendment of TX1 cell activation, toxicity due to the inhibitor alone, or a toxic synergistic effect of the TX1 cells, inhibitor and promutagen. The resolution of the plant cell/microbe coincubation assay is sufficiently high that the effect of μM to mM concentrations of specific inhibitors was easily detected.

3.2.1 Experiments With Diethyldithiocarbamate

Diethyldithiocarbamate, a metal chelator, was titrated (50 μM-50 mM) in coincubation reaction tubes with plant cells, bacterial cells and a constant amount of 50 μM 2-aminofluorene. The inhibition of revertant TA98 colonies was a function of increased diethyldithiocarbamate concentration with 50 percent inhibition between 750 μM and 1 mM. At the lowest concentration of diethyldithiocarbamate (50 μM) a significant inhibition of 2-aminofluorene activation was noted (●). This inhibition could be due to killing of the TX1 (☐) or TA98 (△) cells. However, no consistent decrease in the relative viability of the TX1 or TA98 cells attended the inhibition curve. Diethyldithiocarbamate was not mutagenic (○). In separate studies, diethyldithiocarbamate (25 μM-50 mM) was introduced into reaction tubes with plant cells, bacterial cells and 500 μM *m*-phenylenediamine. The inhibition of revertant TA98 colonies was a function of increased diethyldithiocarbamate concentration with 50% inhibition between 750 μM and 1 mM (●). At a concentration of 250 μM diethyldithiocarbamate, a significant inhibition of the plant activation of *m*-phenylenediamine was noted. No decrease in the relative viability of the TX1 (☐) or TA98 (△) cells attended the inhibition curve (Figure 5).

Figure 5 The effect of diethyldithiocarbamate on the TX1-cell activation of *m*-phenylenediamine and 2-aminofluorene.

3.2.2 Experiments With Metyrapone

Metyrapone is a specific cytochrome P-450 inhibitor in mammals and a weak inhibitor of 2-aminofluorene *N*-hydroxylase. Metyrapone at concentrations below 7.5 mM did not significantly

inhibit the activation of 2-aminofluorene. The activation of 2-aminofluorene was diminished by approximately 50% by 15 mM metyrapone (●). However, at this concentration toxicity was beginning to be expressed in the TX1 cells (□). The reduction of the activation of 2-aminofluorene appears to be due to toxicity in the plant cells (Figure 6). These data illustrate the necessity of monitoring viability when studying the inhibition of activation or mutagenesis. Metyrapone at concentrations above 10 mM was not toxic to TA98 (△) or TX1 (□) and it was not mutagenic (○). These data indicate that metyrapone and 2-aminofluorene may interact synergistically to produce a toxin or that metyrapone may inhibit a step in the TX1 metabolism of 2-aminofluorene that results in a phytotoxic, nonmutagenic intermediate. Metyrapone at concentrations below 7.5 mM did not significantly inhibit the activation of m-phenylenedi-amine. The activation of m-phenylenediamine was diminished by approximately 50% by 15 mM metyrapone (●). At metyrapone concentrations above 1 mM, toxicity to TX1 cells was expressed (□). The reduction of the activation of m-phenylenediamine by TX1 cells appears to be a function of metyrapone toxicity in the plant cells. These data agree with those found using 2-aminofluorene (Figure 6).

Figure 6 The effect of metyrapone on the TX1-cell activation of m-phenylenediamine and 2-aminofluorene.

3.2.3 Experiments With 7,8-Benzoflavone

The capacity of 7,8-benzoflavone to inhibit the activation of 2-aminofluorene was evaluated over a concentration range from 1 μM-2 mM (Figure 7). At concentrations above 1 μM, 7,8-benzoflavone significantly reduced the mean number of TA98 revertants per plate (●). 7,8-Benzoflavone was not a direct acting mutagen and was not activated by plant cells (○). The inhibition curve exhibited a concentration-dependent decrease with increasing 7,8-benzoflavone concentration. The viability of the TX1 (□) and TA98 (△) cells was not affected at concentrations below 750 μM. However, above this concentration the inhibitor was toxic to both cell types. An inhibition of TA98 reversion was observed without concomitant cellular toxicity from 10-250 μM 7,8-benzoflavone. Thus 7,8-benzoflavone at μM concentrations is an effective inhibitor of 2-aminofluorene activation by TX1 cells. The effect of 7,8-benzoflavone on the plant activation of m-phenylenediamine was investigated.
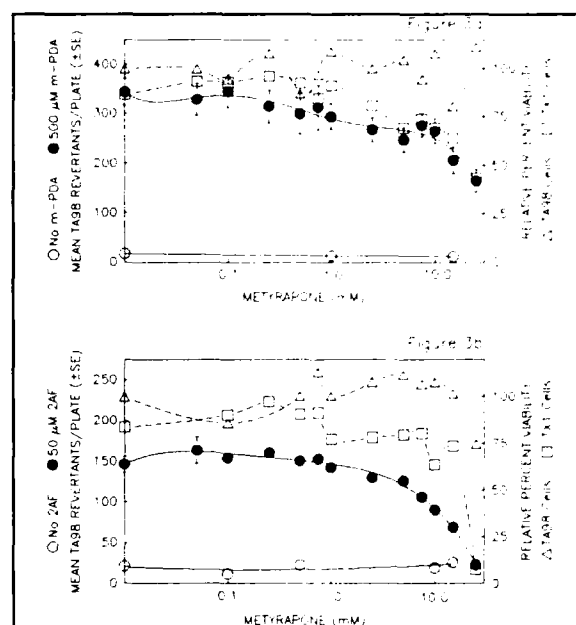

Figure 7 The effect of 7,8-benzoflavone on the TX1-cell activation of m-phenylenediamine and 2-aminofluorene.
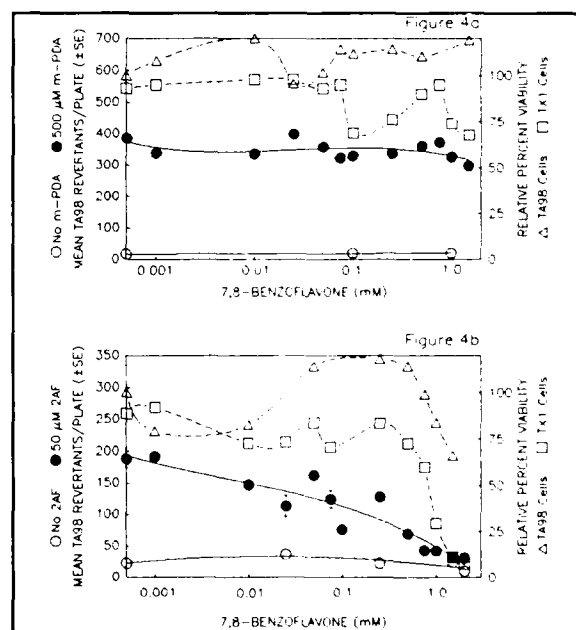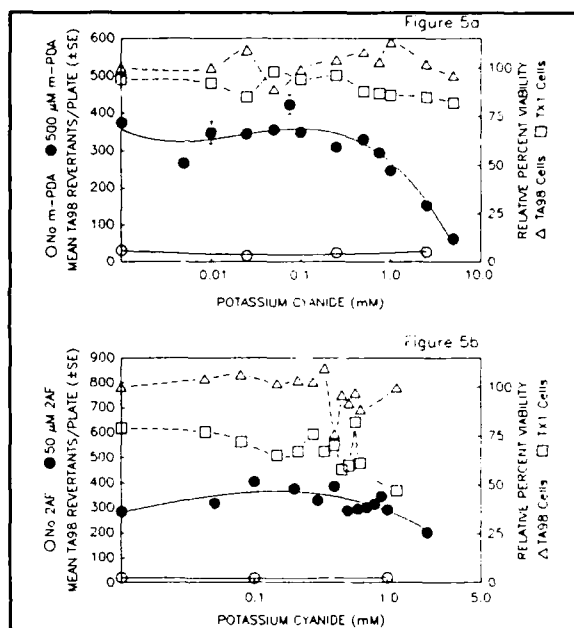
There was i o decrease in the number of TA98 revertants over the entire concentration range of 1 µM to 1.5 mM (●). 7,8-Benzoflavone is not an inhibitor of m-phenylenediamine activation by TX1 cells (Figure 7).

3.2.4 Experiments With Potassium Cyanide

Potassium cyanide is an inhibitor of plant peroxidases. Potassium cyanide was toxic to the TX1 cells at concentrations above 1 mM (□). However, a significant enhancement in the activation of 2-aminofluorene was observed in some reaction tubes from 100 µM to 1 mM (●); within this concentration range, TX1 (□) and TA98 (△) viability was not affected. Prior to its toxic effects, potassium cyanide may be enhancing the plant activation of 2-aminofluorene (Figure 8). However, the great variability of the data and the broad range of cellular effects induced by potassium cyanide preclude a more detailed analysis with this agent. The effect of 5 µM-5 mM potassium cyanide was studied in the activation of m-phenylenediamine. A significant inhibition of mutation induction was observed at potassium cyanide concentrations above 750 µM (●). There was not a corresponding decrease in viability in either cell type (□ △). Thus potassium cyanide inhibited m-phenylenedi-amine activation at nontoxic concentrations (Figure 8).

Figure 8 The effect of potassium cyanide on the TX1-cell activation of m-phenylenediamine and 2-aminofluorene.

3.2.5 Experiments With (+)-Catechin

Concentrations of (+)-catechin from 25 µM-25 mM were titrated against TX1 and TA98 cells with 2-aminofluorene (Figure 9). From 25 µM-2.5 mM, (+)-catechin significantly enhanced the plant activation of 2-aminofluorene into a mutagen (●). The number of TA98 revertants per plate of the positive control was 219.8 with the highest enhancement induced at 750 µM (+)-catechin. (+)-Catechin was not a direct-acting or a plant-activated mutagen and it was not toxic to the bacterial cells (△). The only toxicity to the plant cells was observed at 25 mM (□). (+)-Catechin concentrations above 5 mM significantly inhibited the activation of 2-aminofluorene. Monooxygenase enzymes that hydroxylate the ring carbons of 2-aminofluorene render the agent non-mutagenic. At low concentrations of (+)-catechin, C-hydroxylation might be preferentially inhibited resulting in an enhancement in the TA98 reversion frequency. At higher concentrations of (+)-catechin, the presumed N-hydroxylation of 2-aminofluorene may be inhibited with a decrease in the mutation frequency. (+)-Catechin was titrated against TX1 and TA98 cells with 500 µM m-phenylenediamine (Figure 9). There was no effect on the mean number of TA98 revertants at concentrations below 2.5 mM (●). From 2.5 mM - 25 mM there was a concentration-dependent reduction in the number of revertants. (+)-Catechin concentrations above 10 mM were toxic to TX1 (□) and TA98 (△) cells. From 1 - 10 mM, (+)-catechin inhibited the plant activation of m-phenylenediamine without

any toxicity. There was no enhancement in mutage-
nicity as observed with 2-aminofluorene.

3.2.6 Experiments With Methimazole

Methimazole is a high-affinity flavin-contain-
ing monooxygenase substrate. 50 µM to 25 mM
methimazole was added to TX1 cells, TA98 cells
and 50 µM 2-aminofluorene (Figure 10). Methima-
zole did not inhibit the plant activation of 2-amino-
fluorene (●). At concentrations above 100 µM it
exhibited a significant enhancement of the mutagen-
ic potency of 2-aminofluorene. This enhancement
was much lower than that induced with (+)-cate-
chin. Methimazole was not directly mutagenic (○)
or toxic (□ △). These data suggest the same
mechanism of action suggested for (+)-catechin
with the inhibition of detoxification enzymes result-
ing in more 2-aminofluorene available for activation.
Concentrations of methimazole from 50 µM to 25
mM were added to TX1 cells, TA98 cells and 500
µM m-phenylenediamine. Methimazole at concen-
trations above 2.5 mM inhibited the plant activation
of m-phenylenediamine (●). No toxic effects to
the TX1 (□) or TA98 (△) cells were observed.
These data indicate that a flavin-containing mono-
oxygenase may be involved in the plant activation of
m-phenylenediamine.

Figure 9 The effect of (+)-catechin on the TX1-cell
activation of m-phenylenediamine and 2-aminofluorene.

3.2.7 Experiments With Acetaminophen

Acetaminophen is a competitive inhibitor of
peroxidase. There was inhibition in the plant
activation of 2-aminofluorene at concentrations of
acetaminophen above 10 mM (●) (Figure 11).
Acetaminophen alone was not mutagenic (○) or
toxic (□ △). These data suggest that tobacco cell
peroxidase is a minor pathway in the activation of
2-aminofluorene. Contrasting results were obtained
with acetaminophen and m-phenylenediamine.
Concentrations of acetaminophen above 2.5 mM
caused a concentration-dependent decrease in the
mutation frequency (●). There was no decrease in
viability for either cell type (□ △). Concentrations

Figure 10 The effect of methimazole on the TX1-cell
activation of m-phenylenediamine and 2-aminofluorene.

of acetaminophen that significantly inhibited the activation of m-phenylenediamine by TX1 cells also
inhibited the activity of cellular peroxidase in the same cells. These data suggest that tobacco cell
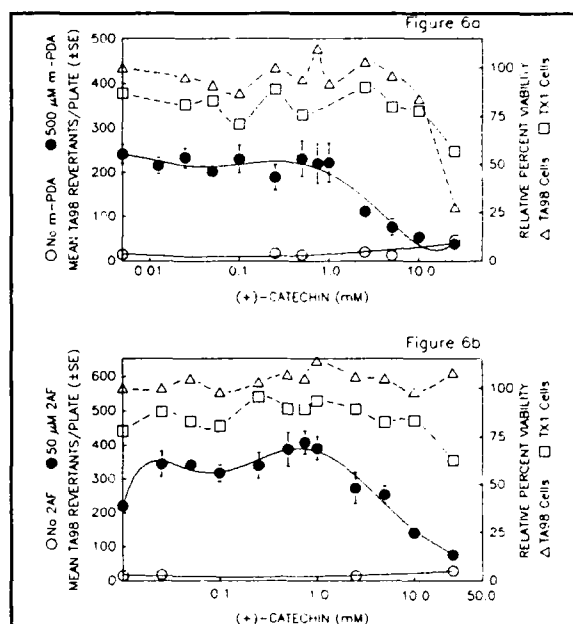peroxidases function as a major pathway for the plant activation of m-phenylenediamine.

This study illustrates that by using specific enzyme inhibitors the plant cell/microbe coincubation assay can be employed to investigate biochemical mechanisms of plant activation. The activation of 2-aminofluorene by TX1 cells was governed by an enzyme system(s) that was inhibited by μM amounts of diethyldithiocarbamate or 7,8-benzoflavone. (+)-Catechin (at low concentrations) or methimazole enhanced the activation of 2-aminofluorene while higher concentrations of (+)-catechin were inhibitory. A significant pathway of the plant activation of 2-aminofluorene is via a cytochrome P-448-dependent N-hydroxylase. Peroxidase may be a minor pathway. The presence of a FAD-dependent monooxygenase in the activation of 2-aminofluorene was not detected. The activation of m-phenylenediamine was inhibited by μM amounts of diethyldithiocarbamate and mM amounts of (+)-catechin, methimazole, potassium cyanide, and acetaminophen. There was no enhancement effect observed with (+)-catechin. This may be due to the different biochemical pathways involved in

Figure 11 The effect of acetaminophen on the TX1-cell activation of m-phenylenediamine and 2-aminofluorene.

activation. Inhibition by methimazole indicates the presence of a FAD-dependent monooxygenase in the activation of m-phenylenediamine. The inhibition of the plant activation of m-phenylenediamine by potassium cyanide and acetaminophen indicates that peroxidases may function as a major pathway. The experiments with metyrapone and potassium cyanide illustrate the importance of measuring not only the altered mutation induction frequency, but also the viability of the activating system and the genetic indicator organism to eliminate artifacts due to toxicity. Without viability as an endpoint, no study on the inhibition of mutagenesis or activation can be considered complete.

3.3   The Investigation That Specific Inhibitors That Repress the Activation of Promutagens Function by Competitive or Noncompetitive Inhibition.

We are currently investigating the biochemical mechanisms of plant activation. It has been shown that the compound diethyldithiocarbamate inhibited the metabolism of promutagens in both animal systems (Masuda and Nakayama, 1982) and plant systems (Gichner and Veleminsky, 1984; Gichner et al., 1988). Recently we discovered that diethyldithiocarbamate inhibited the tobacco cell activation of 2-aminofluorene and m-phenylenediamine. By using specific enzyme inhibitors we reported indirect evidence that tobacco cell peroxidases were involved in the activation of these aromatic amines (Wagner et al., 1989; 1990). The purpose of this research was to determine if diethyldithiocarbamate suppressed the tobacco cell activation of aromatic amines by inhibiting cellular peroxidases.

3.3.1 Plant Cells and Plant Cell Homogenates

Long-term plant cell suspension cultures of tobacco (*Nicotiana tabacum*) cell line TX1 were maintained in MX medium, a modified liquid culture medium of Murashige and Skoog (1962). For each experiment, plant cells from a 7-day culture were harvested, washed, and adjusted to 100 mg fresh weight/ml in MX⁻ medium. These cells were treated with diethyldithiocarbamate (0 - 25 mM) at 28°C for 1 h with shaking (150 rpm). After treatment the cells were centrifuged, the supernatant was decanted, and the cells were suspended. Each cell suspension was homogenized with a PolyTron tissue disrupter for 45 sec at 4°C, and the cell debris was removed by centrifugation at 15,000 × g for 2 min. An aliquot of the supernatant was frozen at -80°C for later protein analysis. The other portion was kept on ice and immediately analyzed for peroxidase activity.

3.3.2 Determination of Peroxidase Activity

To determine peroxidase activity we measured the oxidation of guaiacol to tetraguaiacol by monitoring the change in absorbance at 470 nm (Maehly and Chance, 1954). Peroxidase activity was analyzed in a reaction volume of 3 ml containing 50 mM potassium phosphate buffer, pH 7.0, 100 μl of 0.3% H_2O_2, 1 ml of a 1% guaiacol solution, and 25 μl of the TX1 cell homogenate. The cuvettes used as blanks were identical except that MX⁻ medium was used instead of homogenate. Peroxidase activity was measured over a 5 min time period using a model 552A Perkin-Elmer double-beam spectrophotometer at 470 nm. Three independent replicates were conducted for each measurement within each experiment.

3.3.3 *Assay for Protein Concentration*

3.3.3.1 Growth Curve and Protein Content of TX1 Cells

In order to investigate the biochemical mechanism of plant activation, a growth curve was established for these TX1 cells. Several flasks were inoculated with 3 g each from a 7-day culture. At approximately 24-hour intervals, fresh weight was measured from three 25-ml samples, by drying the cells under vacuum, and weighing the cells. These cells were then titred by suspending them to a final concentration of 100 mg/ml solution in distilled water. Protein was extracted from this solution by shearing the cells with a PolyTron homogenizer and centrifuging at 10,000 × g for two min to remove cellular debris. The supernatant was then assayed for protein content using the Bio-Rad protein assay. The growth curve revealed that TX1 cells remain in lag phase for three to four days followed by log growth for approximately three to four days, reaching stationary phase around day 7. The protein content of TX1 cells, however, did not coincide with their growth curve. Instead, the protein increased quickly with
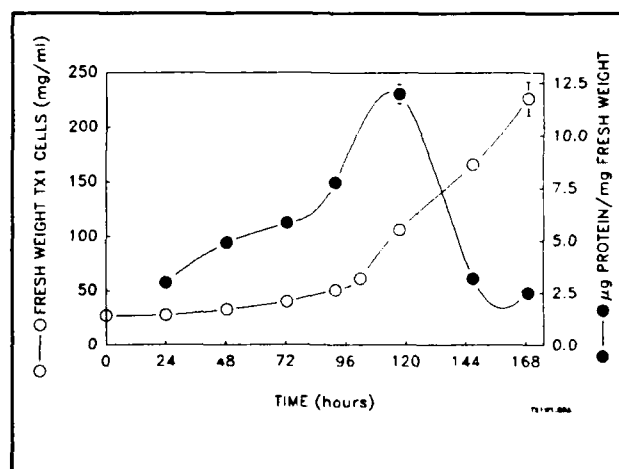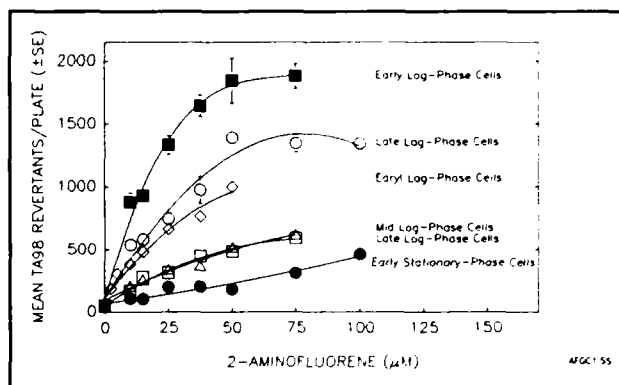

Figure 12 Relationship between the growth curve of TX1 cells and protein content

a maximum content during log phase followed by a sharp decrease back to base levels during stationary phase.

3.3.3.2 Differential Activation With 2-Aminofluorene

Previous studies suggested that activation by TX1 cells of m-phenylenediamine and 2-aminofluorene depend on the growth stage of the cell culture. It was demonstrated using the plant cell/microbe coincubation assay that 2-aminofluorene is best activated by cells in early log phase, while m-phenylenediamine activation peaks in mid- to late-log phase. The age of TX1 cells is directly related to the production of peroxidases, with peroxidase levels increasing with the growth of the cells. The level of activation on different days of the growth curve was determined for the promutagen 2-amino-fluorene. On days 1 through 7, activation was measured and was found to directly relate to the levels of protein in the cells. On day 3, when the cells are just entering log phase, the highest degree of activation was attained, with

Figure 13 The effect of cell growth stage on the plant activation of 2-aminofluorene as measured by the number of TA98 revertants/plate

approximately 1800 TA98 revertants per plate (75 µM 2-aminofluorene). Day 2 cells yielded the second highest activation rate, followed by day 1 cells. Cells from days 4 and 5 showed activation levels below that of day 1 cells. Day 7 cells activated 75 µM 2-aminofluorene resulting in only 400 TA98 revertants per plate, the lowest level of activation. The maximum rate of activation of this promutagen correlates with the maximum protein content of the TX1 cells. Activation of 2-aminofluorene does not correspond with the peroxidase activity in the plant cells.

The activation of this promutagen, 2-aminofluorene, appears to differ from that of the promutagen m-phenylenediamine in that the activation of m-phenylenediamine is directly related to peroxidase activity, rather than protein content. Although this has not specifically been shown in this TX1 culture due to loss of these cells in a power failure, other studies have shown this to be true. In terms of the mechanism of activation, this suggests that two different genes may be involved in the activation of these two promutagens, one being turned on in the early stages of growth (2-aminofluorene) and the other in the later stages of growth (m-phenylenediamine).

3.3.3.3 Protein Content in Fresh Versus Frozen Cells

When engaging in lengthy studies involving cell suspension cultures, such as growth curves, it would be convenient to store samples for extended periods of time to analyze all at once. For example, the Bio-Rad protein assay requires that a standard protein curve be run with every assay. Being able to freeze and store samples (TX1 cells) until the end of a study would lessen the amount of time, work, and supplies used. Because of the ease of preparation, fresh 7-day TX1 cells were used. The fresh weight of the cells was titred to 100 mg/ml. The titred solution was divided into two samples. One sample was sheared with a PolyTron homogenizer, centrifuged at 10,000 × g for two minutes, and the resulting supernatant was analyzed in a dose-dependent manner to determine protein content as described previously using the Bio-Rad assay. The other sample was frozen in

a -80°C freezer for a minimum of 24 h before being thawed, sheared, centrifuged, and analyzed in the same manner. Less protein was extracted from th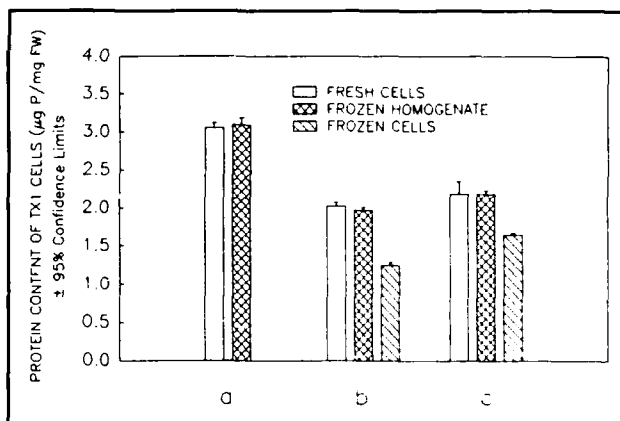e frozen cells than from the fresh preparation. This was contrary to our hypothesis that freezing and thawing cells would cause additional disruption of the cells and allow more protein to be extracted from frozen cells than from fresh cells. The experiment was repeated, yielding the same result. The protein content from the frozen cell preparations had much greater variance than did the fresh cell preparations. This suggests that freezing the cells prior to shearing, centrifuging, and measuring protein content not only reduces the amount of protein extracted, but is also an unreliable measure of the true value.

Figure 14 Comparison of protein content in fresh versus frozen 7-day TX1 cells

3.3.3.4 Protein Content in Fresh Versus Frozen Cell Homogenates

For the Bio-Rad protein assay, it would be convenient to obtain samples which could be frozen for future analysis. A seven day old TX1 culture was titred to 100 mg/ml fresh weight, sheared, and centrifuged as before. This was divided into two samples. One sample was analyzed for protein content immediately using the Bio-Rad protein assay. The other sample was frozen in a -80°C freezer for a minimum of 24 hours before thawing and analyzing. Ten samples of 35 µl each were analyzed for protein content from both groups. Three independent experiments were run, and in all three cases, the protein content of the frozen cell homogenate did not significantly differ from that of fresh cells. In two of the three experiments, a portion of the initial titred cell suspension was removed prior to shearing, was frozen, thawed, sheared, centrifuged, and analyzed for protein content. In both cases, significantly less protein was extracted from the frozen cells, confirming our previous study. It can be concluded that freezing the cell homogenate, rather than the whole cells, does not affect protein content of TX1 cell cultures.


Figure 15 Comparison of protein content in 3 different 7-day TX1 cells preparations. *a*, *b*, and *c* represent data collected from three independent experiments

3.3.4 Inhibition of TX1 Cell Activation by Diethyldithiocarbamate

Diethyldithiocarbamate inhibited the TX1 cell activation of m-phenylenediamine (Figure 5). A significant decline in activation occurred above 75 μM diethyldithiocarbamate with 50% inhibition between 1 and 1.5 mM. Diethyldithiocarbamate was an excellent inhibitor of the TX1 cell activation of 2-aminofluorene (Figure 5). A significant decrease in the number of TA98 revertants occurred with 50 μM diethyldithiocarbamate with 50% inhibition observed between 750 μM and 1 mM. With both promutagens there was no decrease in the viability of either TX1 or TA98 cells.

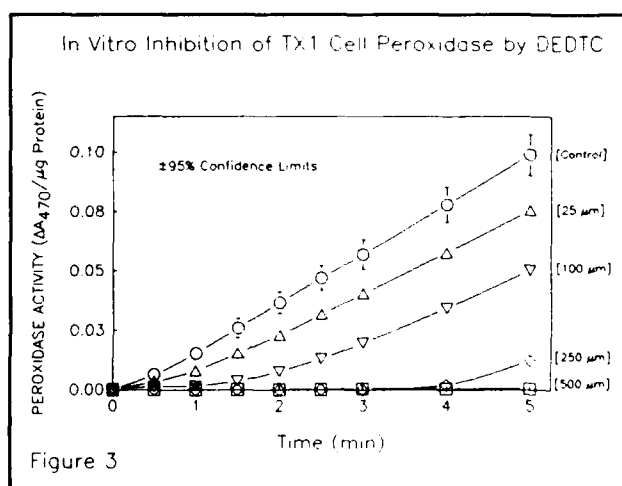### 3.3.5 In Vivo Inhibition of TX1 Cell Peroxidase by Diethyldithiocarbamate

Intact TX1 cells were exposed *in vivo* to diethyldithiocarbamate concentrations from 250 μM - 25 mM in four separate experiments. TX1 cell homogenates were prepared and both peroxidase activity and protein content were measured. One experiment is presented in Figure 16. TX1 cells exposed to diethyldithiocarbamate express reduced peroxidase activities when normalized on a protein basis. The diethyldithiocarbamate concentrations which caused a 50% reduction in TX1 cell peroxidase activity (750 μM - 2.5 mM) also caused a 50% inhibition of the TX1 cell activation of m-phenylenediamine and 2-aminofluorene.
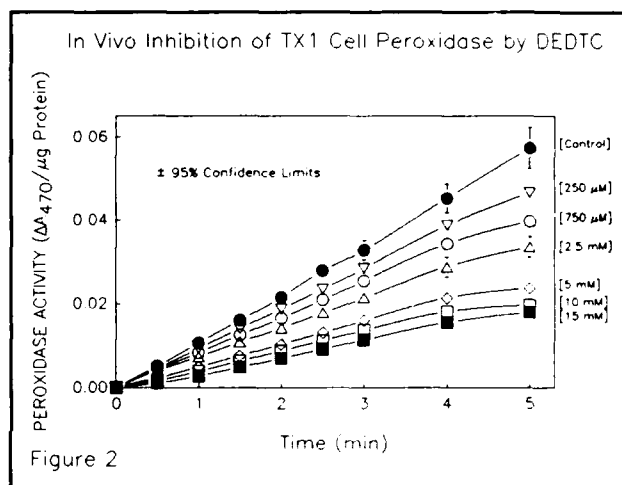
Figure 16 *In Vivo* inhibition of TX1 cell peroxidase by diethyldithiocarbamate

3.3.6 In Vitro Inhibition of TX1 Cell Peroxidase and Horseradish Peroxidase by Diethyldithiocarbamate

The *in vitro* inhibition of TX1 cell peroxidase was determined by adding diethyldithiocarbamate (25 - 750 μM) directly to TX1 cell homogenates in five independent experiments. A concentration-dependent reduction in peroxidase activity was observed throughout the entire concentration range. The data from one experiment are presented in Figure 17. To confirm that diethyldithiocarbamate can inhibit peroxidase enzymes, two experiments were conducted using pure horseradish peroxidase. A concentration-dependent reduction in the activity of horseradish peroxidase was observed (data not shown).

Figure 17 *In Vitro* inhibition of TX1 cell peroxidase by diethyldithiocarbamate

3.3.7 Kinetics of TX1 Cell Peroxidase

TX1 cells were incubated with and without 750 μM diethyldithiocarbamate. Separate TX1 cell homogenates were prepared under identical conditions. The peroxidase activities of both homogenates were measured using different concentrations of substrate (H_2O_2). Non-competitive inhibition was indicated by identical K_m values for both control and diethyldithiocarbamate-treated cells. From three independent experiments, the K_m values for the control and treated cells were 2.79 ± 0.50 mM and 2.31 ± 0.27 mM, respectively. The data from one experiment are presented in a Lineweaver-Burk plot (Figure 18).
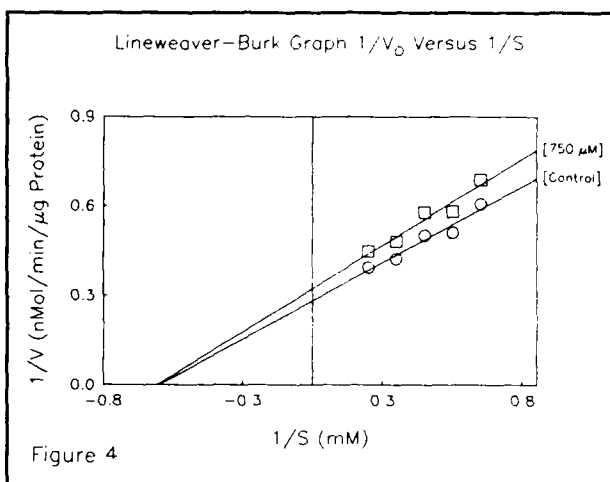
Figure 4

Figure 18 Lineweaver-Burk analysis of the inhibition of TX1-cell peroxidase by diethyldithiocarbamate

From the above studies we concluded that diethyldithiocarbamate suppressed the tobacco cell activation of m-phenylenediamine and 2-aminofluorene. Diethyldithiocarbamate was a potent inhibitor of tobacco cell peroxidase activity under in vivo and in vitro conditions. Kinetic experiments demonstrated that diethyldithiocarbamate was a non-competitive inhibitor of TX1 cell peroxidase. One mechanism for the antimutagenic effect of diethyldithiocarbamate was through its inhibition of cellular peroxidases.

4 LIST OF PUBLICATIONS

4.1 Published Abstracts (Peer Reviewed)

Wagner, E.D., M.M. Verdier and M.J. Plewa. 1989. Comparison of the plant cell activation of two promutagens using enzyme inhibitors. Environ. Molecular Mutagenesis 14:211, Suppl. 15.

Smith, S.R., M.M. Verdier, E.D. Wagner and M.J. Plewa. 1989. Protein content of tobacco cells in relation to the plant activation of m-phenylenediamine and 2-aminofluorene. Environ. Molecular Mutagenesis 14:188-189, Suppl. 15

Plewa, M.J. 1989. The activation of promutagens by plant cell systems. (Invited symposium lecture to the Fifth International Conference on Environmental Mutagens). Environ. Molecular Mutagenesis 14:154, Suppl. 15

Wagner, E.D., S.R. Smith, K. Hajek and M.J. Plewa. 1990. The mechanism of the antimutagenic effect of diethyldithiocarbamate in the plant activation of promutagens. Environ. Molecular Mutagenesis 15:62, Suppl. 17.

Plewa, M.J. and E.D. Wagner. 1990. The biochemical basis of the activation of promutagens by plant cell systems. Environ. Molecular Mutagenesis 15:48, Suppl. 17.

4.2   Published Papers (Peer Reviewed)

Wagner, E.D., J.M. Gentile, and M.J. Plewa. 1989. Effect of specific monooxygenase and oxidase inhibitors on the activation of 2-aminofluorene by plant cells. Mutation Research 216:163-178.

Plewa, M.J. 1990. Activation of promutagens by plant cell systems. Proceedings of the Fifth International Conference on Environmental Mutagens. IN PRESS.

Plewa, M.J. 1990. Basic and Applied Research on the Activation of Promutagens by Plant Cell Systems. Proceedings of the Fifth International Conference on Environmental Mutagens – Symposium on Genetic Toxicology in Developing Countries. IN PRESS.

Wagner, E.D., M.M. Verdier and M.J. Plewa. 1990. The biochemical mechanisms of the plant activation of promutagenic aromatic amines. Environmental and Molecular Mutagenesis 15: IN PRESS

Plewa, M.J. 1990. The Biochemical Basis of the Activation of Promutagens by Plant Cell Systems, Invited publication ASTM

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5.4   Degrees Awarded

Ms. Kathryn L. Hajek, B.S. May 1990, Senior Thesis Title, "The Effect of Diethyldithiocarbamate on Plant Cell Peroxidase."

Ms. Elizabeth D. Wagner, M.S. May 1989, Thesis Title, "Effects of Specific Monooxygenase and Oxidase Inhibitors on the Activation of 2-Aminofluorene by Plant Cells," 96 pp.

Ms. Shannon Smith, M.S. May 1990, Thesis Title, "Studies with *Nicotiana tabacum* and *Selenastrum capricornutum* that Lead to the Biochemical Mechanisms of the Plant Activation of *m*-Phenylenediamine and 2-Aminofluorene," 87 pp.

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